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ORIGINAL ARTICLE

# A single substitution in amino acid 184 of the NP protein alters the replication and pathogenicity of H5N1 avian influenza viruses in chickens

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**Abstract** Changes in the NP gene of H5N1 highly pathogenic avian influenza (HPAI) viruses have previously been shown to affect viral replication, alter host gene expression levels and affect mean death times in infected chickens. Five amino acids at positions 22, 184, 400, 406, and 423 were different between the two recombinant viruses studied. In this study, we individually mutated the five amino acids that differed and determined that the difference in virus pathogenicity after NP gene exchange was a result of an alanine to lysine change at position 184 of the NP protein. Infection with viruses containing a lysine at NP 184 induced earlier mortality in chickens, increased virus titers and nitric oxide levels in tissues, and resulted in up-regulated host immune genes, such as α-interferon (IFN-α), γ-interferon (IFN-γ), orthomyxovirus resistance gene 1 (*Mx1*), and inducible nitric oxide synthase (iNOS). This study underlines the importance of the NP in avian influenza virus replication and pathogenicity.

## Introduction

Infection with H5N1 highly pathogenic avian influenza (HPAI) viruses in poultry causes devastating disease, resulting in massive poultry losses worldwide. A well-known virulence factor identified for HPAI viruses is the presence of multiple basic amino acids at the cleavage site of the hemagglutinin protein [5, 6, 23, 24, 26, 42]. The non-structural protein (NS1) has also been shown to influence the outcome of virus infection through its modulation of the interferon pathways, influencing the outcome of viral infection [7, 18, 30]. Replication of the single-stranded, negative-sense RNA influenza viruses is dependent on four viral proteins, including the polymerase proteins (PB1, PB2 and PA) and the nucleoprotein (NP), which binds the RNA to form the functional viral ribonucleoprotein (vRNP) complex [36]. The overall efficiency of influenza virus replication can also contribute to virulence, and mutations or reassortment can disrupt the core polymerase components, affecting its function [14, 25, 33, 39].

The NP protein is not only important for influenza virus replication, but many other cellular binding partners have been identified that interact with NP, including chromosome region maintenance (CRM1) [13], filamentous actin [10], and the RNA helicase BAT1/UAP56 [32]. One of these binding partners, importin-α, helps transport NP and vRNPs into the nucleus due to the presence of nuclear localization signals (NLS) [49, 52]. The NP/importin-α interaction has recently been implicated in the host-range restriction of influenza viruses in mammalian cells [15]. Reassortment studies have shown that NP is also involved in species restriction in squirrel monkeys [8, 44, 46], although the mechanisms by which it does so are unclear.

In an earlier study, the virulence determinants of several H5N1 HPAI reassortant viruses in chickens were identified [51]. Exchanging the NP genes from the reverse-genetic HPAI virus, rEgret (a variant derived from A/Egret/HK/757.2/02), for the NP gene of rIndo (a variant derived from A/Ck/Indonesia/7/03) in the rIndo background, resulted in increased replication, altered host gene expression and decreased mean death times (MDTs) compared to infection with the rIndo virus in chickens. Five amino acid differences were found to exist between the NP of rEgret and

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rIndo viruses (amino acids 22, 184, 400, 406, and 423). The rIndo virus, although pathogenic, had decreased pathogenicity and replication in chickens compared to the wild-type A/Ck/Indonesia/7/03 virus. Sequence comparison to the parent virus revealed a mutation at position 184 of the NP protein, as found in the rEgret NP, as well as a mutation in NS1 (G148E) and HA (I194V). In this study, we systematically changed each of the five amino acids that were different from the rEgret NP or reconstituted the altered NP and NS genes of the rIndo virus to the sequence of the wild-type A/Ck/Indonesia/7/03 virus in order to determine which amino acids were responsible for the differences in pathogenicity observed among these viruses. The HA I194V mutation was not changed in this study, as this amino acid was not anticipated to be a causative mutation for the difference in pathogenicity of the viruses observed.

## Materials and methods

### Viruses and cells

The H5N1 highly pathogenic avian influenza (HPAI) virus A/Ck/Indonesia/7/03 was isolated from diagnostic samples at Southeast Poultry Research Laboratory and was propagated in embryonating chicken eggs (ECEs). The H5N1 HPAI A/Egret/HK/757.2/02 virus was kindly provided by Trevor Ellis, Agriculture Fisheries and Conservation Department, Hong Kong, to Southeast Poultry Research Laboratory. All experiments using H5N1 HPAI viruses were conducted using biosafety level 3 Ag (BSL-3 Ag) containment at SEPRL, USDA in Athens, GA, and all personnel were required to wear a powered air protection respirator with a high-efficiency particulate air (HEPA)-filtered air supply (3M$^{TM}$, St. Paul, MN). Cells were maintained in Dulbecco's modified essential medium (DMEM) + 10% FBS (Invitrogen Inc., Carlsbad, CA). The cells used in the experiments were: primary chicken embryo fibroblasts (CEFs) isolated from 10-day-old embryos and maintained at 37°C; DF-1 cells (immortalized chicken embryo fibroblast cell line) grown at 39°C; and 293T cells (human embryonic kidney 293 cells) grown at 37°C.

### Generation of recombinant viruses by reverse genetics

The H5N1 HPAI reverse-genetic viruses, rIndo and rEgret, were derived from A/Ck/Indonesia/7/03 and A/Egret/HK/757.2/02 as described previously [51]. A comparison of the amino acid sequences of the recombinant viruses is shown in Table 1. The recombinant viruses used in this study were obtained as follows: site-directed modification of the NS (amino acid 148) and NP (amino acids 22, 184, 400, 406, and 423) gene transcription plasmids used to generate rIndo was performed using a modified two-stage PCR mutagenesis procedure as described previously [50]. Table 2 lists the recombinant viruses that were generated. Amino acid mutations are as follows: rIndo/NP 22A (T → A), rIndo/NP-184K (A → K), rIndo/NP 400R (K → R), rIndo/NP 406V (I → V), rIndo/NP 423S (A → S), and rIndo/NS-148E (G → E). Transcription and expression plasmids were constructed as described previously [34]. In brief, 293T cells were transfected with 1 μg of each of the eight transcription and four protein expression plasmids and 11 μl of Lipofectamine 2000 (Invitrogen) in a 2-ml volume of OptiMEM-I (Invitrogen). Cells were washed after 4 h at 37°C, and the medium was replaced with DMEM + 10% FBS (Invitrogen) for 72 h. ECEs were inoculated with 100 μl of the cell supernatant. Virus was harvested from the allantoic fluid of eggs 36–48 h after inoculation and titrated in ECEs. Titration endpoints were calculated by the method of Reed and Muench [38]. Variant viruses are variations of the wild-type virus resulting from recombinant virus creation that did not result in identical pathogenicity as the wild-type virus.

**Table 1** Amino acid sequence comparisons of A/Ck/Indonesia/7/03 and recombinant viruses

| Gene product | Amino acid | Virus | | |
|---|---|---|---|---|
| | | A/Ck/Indonesia/7/03 | rIndo | rEgret |
| NS | 148 | E | G | E |
| NP | 22 | T | T | A |
| | 184 | K | A | K |
| | 400 | K | K | R |
| | 406 | I | I | V |
| | 423 | A | A | S |

**Table 2** Mean death times of 2-week-old infected chickens

| Virus | No. of sick/dead (total) | MDT (days) |
|---|---|---|
| A/Ck/Indonesia/7/03 | 8/8 (8) | 1.5[a] |
| rIndo | 6/6 (8) | 5.6 |
| rIndo/NP 22A | 5/3 (8) | 5.6[b] |
| rIndo/NP-184K | 8/8 (8) | 2.0[a,c] |
| rIndo/NP 400R | 6/6 (8) | 5.8[b] |
| rIndo/NP 406V | 6/4 (8) | 4.5[b] |
| rIndo/NP 423S | 6/5 (8) | 5.2[b] |
| rIndo/NS-148E | 8/5 (8) | 5.2[b] |
| rIndo/NS-148E/NP-184K | 8/8 (8) | 1.87[a,c] |

[a] MDT is significantly different than rIndo

[b] MDT is not significantly different than rIndo

[c] MDT is not significantly different than A/Ck/Indonesia/7/03

### Sequencing of influenza virus genes

Confirmatory sequencing of the rescued rIndo and rEgret viruses was performed previously [51]. The mutated gene transcription plasmids were fully sequenced in order to confirm the presence of the intended mutations. ABI Big Dye Terminator version 1.1 sequencing kit (Applied Biosystems, Foster City, CA) run on a 3730 XL DNA Analyzer (Applied Biosystems) sequencer was used for sequencing PCR products. The MegAlign program (Lasergene 7.1, DNASTAR, Madison, WI) was used to compare nucleotide sequences, using the Clustal W alignment algorithm.

### In vivo characterization of recombinant viruses

In order to determine the pathogenic phenotypes of the viruses in chickens, two-week-old specific-pathogen-free (SPF) White Leghorn chickens (*G. gallus domesticus*) (from Southeast Poultry Research Laboratory in-house flocks) were inoculated intranasally (IN) with the recombinant viruses and were examined twice a day for the presence of clinical signs for a total of 10 days. The wild-type virus, A/Ck/Indonesia/7/03, was also included for comparison. The birds were housed in self-contained isolation cabinets that were ventilated under negative pressure with HEPA-filtered air and maintained under continuous lighting. The birds had ad libitum access to feed and water. Each group, containing 12 birds, was inoculated IN with 0.1 ml of an inoculum containing $10^6$ $EID_{50}$ of a virus. At 1 day post-inoculation (dpi), blood was drawn from four birds in each group before they were euthanized and necropsied. Gross lesions were recorded, and tissues (lung and spleen) were collected separately from three birds for virus isolation. Lungs, bursa, kidneys, adrenal gland, gonads, thymus, thyroid, brain, liver, heart, ventriculus, pancreas, intestine, spleen, gonads, trachea, and thigh muscle were collected in 10% neutral buffered formalin solution from the same birds. Sample birds, moribund birds, and all birds remaining at the end of a 10-day period were humanely euthanized. MDTs were calculated by determining the sum of the day of death for the chickens and dividing by the total number of dead chickens.

### Histopathology and immunohistochemistry (IHC)

Collected tissues fixed by submersion in 10% neutral buffered formalin were routinely processed and embedded in paraffin. Sections were made at 5 μm and were stained with hematoxylin and eosin (HE). A duplicate 4 μm section was immunohistochemically stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A 1:2,000 dilution of a mouse-derived monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein (developed at Southeast Poultry Research Laboratory, USDA) was applied and allowed to incubate for 2 h at 37°C. The primary antibody was then detected by the application of biotinylated goat anti-mouse IgG secondary antibody using a biotin–streptavidin detection system (Supersensitive Multilink Immunodetection System, Biogenex). Fast Red TR (Biogenex) served as the substrate chromagen, and hematoxylin was used as a counterstain. All tissues were systematically screened for microscopic lesions. Lesions were scored as follows: $-$ = no lesions; $+$ = mild; $++$ = moderate; $+++$ = severe lesions. The intensity of viral antigen staining in each section was scored as follows: $-$ = no antigen staining; $+$ = infrequent; $++$ = common; $+++$ = widespread staining.

### Virus isolation and titrations

Portions of the spleen and lung from three birds per group were collected at 1 dpi in brain heart infusion medium (BHI) (BD Bioscience, Sparks, MD) and stored frozen at −70°C. Titers of infectious virus were determined as follows: tissues were homogenized (10% wt/vol) and diluted in BHI (BD Bioscience) to a final volume of 200 μl. Dilutions of the tissue homogenates were incubated with DF-1 cells in 96-well plates. The presence of virus was determined by positive results scoring for cytopathic effect (CPE). Titration endpoints were calculated by the method of Reed and Muench [38] and reported as tissue culture infectious doses 50% ($TCID_{50}$). The threshold of detection was 1.5 $\log_{10}$ $TCID_{50}$/g tissue.

### Virus growth in chicken embryo fibroblasts

The growth curves of the viruses were determined by virus titration of cell culture supernatants at different time points after infection of primary CEFs. Briefly, $2 \times 10^5$ CEFs were infected with each virus at a multiplicity of infection (MOI) of 0.005 in DMEM + 10% FBS (Invitrogen Inc.). MOI was calculated as follows: MOI of 0.005 = (0.005 × number of cells)/virus titers (as $EID_{50}$). Following adsorption for 1 h at 37°C, non-adsorbed viruses were removed, and the medium was replaced. At 0, 12, 24, 36, and 48 h post-infection, supernatants were collected and stored at −80°C until used for titrations. Virus titers were determined by CPE. The $TCID_{50}$ assay endpoints were calculated by the method of Reed and Muench [38]. Titers were determined in triplicate, and virus titers obtained at the 24 and 36 h time points were analyzed statistically by one-way ANOVA (GraphPad Software Inc., San Diego, CA).

### Total cellular RNA isolation

Total cellular RNA for evaluation of gene expression was prepared from spleen or lung tissues of chickens collected at 1 dpi. Tissue samples were homogenized in 3 ml Minimal Essential medium, Alpha 1× (Invitrogen) by passing the tissues through 100-μm cell strainers (BD Bioscience), and 2 ml of the homogenate was added to 6 ml Trizol (Invitrogen), mixed and stored at −70°C. After thawing, chloroform (1.6 ml) was added to the Trizol/homogenate mixture, mixed and spun at 3,500 rpm for 12 min. The aqueous phase was added to an equal volume of 70% ethanol. RNA midi-prep kits (Qiagen, Valencia, CA) were used to isolate the RNA from the aqueous/ethanol solution and 25 ng of total RNA from each of the four chickens per group was pooled prior to RT-PCR analysis (100 ng total pooled RNA per group was used). RNA was quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

### Semi-quantitative analysis of mRNA gene expression

Reverse transcription PCR was carried out using a Qiagen OneStep PCR kit (Qiagen) according to manufacturer's instructions using 100 ng of pooled cellular RNA for each infected group at 1 dpi in a 25 μl reaction volume ($n = 1$). The primers for IFN-α, IFN-γ, *Mx1*, β-actin, and iNOS were described previously [40]. β-actin served as an amplification and loading control. Bands were visualized by ethidium bromide gel electrophoresis and quantitated using an EDAS 290 imaging station (Kodak, New Haven, CT). Band intensity was normalized to the β-actin control for each sample, and control values were set to 1.

### Nitric oxide assays

Total nitrate and nitrite content produced by NOS was measured in serum samples taken at 1 dpi. Serum was passed through a 10-kDa ultrafilter (Millipore Corp., Billerica, MA) prior to analysis. Total protein in the filtered serum was measured using a DC Protein Assay (Bio-Rad, Hercules, CA). A Nitric Oxide Synthase Assay kit (Calbiochem, Gibbstown, NJ) was used according to the manufacturer's specifications on 40 μl of filtered serum. Values were normalized to the total protein in the filtered serum.

### Statistical analyses

Data were analyzed using Prism v.5.01 software (GraphPad Software Inc.), and values are expressed as the mean ± SEM. The Kaplan–Meier survival rate data were analyzed using the Log-Rank test. One-way ANOVA with Tukey post-test was used to analyze MDTs and virus titers. Statistical significance was set at $p < 0.05$.

## Results

### Amino acid differences in the NP and NS1 proteins of the AIVs

Previously, it was shown that exchanging the NP genes from the rEgret virus (a variant derived from A/Egret/HK/757.2/02) for the NP gene of rIndo (a variant derived from A/Ck/Indonesia/7/03) in the rIndo background resulted in increased replication, altered gene expression and decreased MDTs compared with the rIndo virus in chickens [51]. Comparison of the rIndo NP amino acid sequence with the NP amino acid sequence of the rEgret virus revealed differences in amino acids 22, 184, 400, 406 and 423 (Table 1) [51]. In addition, the rIndo virus resulted in decreased pathogenicity and replication in chickens compared to the wild-type A/Ck/Indonesia/7/03 virus from which it was derived. Infection of chickens with rIndo resulted in a MDT 4.1 days longer than that of the wt Indo virus (Table 2). Sequence analysis of these viruses revealed a mutation at NP 184 and also NS 148, amino acids that differed from the wt Indo virus (Table 1).

### A lysine residue at amino acid 184 of the nucleoprotein increased mortality and decreased the MDT of rIndo in chickens

Using the sequence information above, single amino acid mutations were created in either the NP or NS of the rIndo virus to reassemble the wt Indo virus. Alternatively, single amino acids of the five residues of the NP protein that were different between rEgret and rIndo were altered in order to determine which specific amino acids affected the pathogenicity of the viruses. Table 2 shows the morbidity, mortality and MDTs of 2-week-old chickens infected with each of the mutated viruses we rescued. The rIndo variant had an MDT of 5.6 days compared to the 1.5 days of the wt Indo parent virus from which it was derived and also resulted in less than 100% mortality. The rIndo/NP-184K and rIndo/NS-148E/NP-184K rescued viruses resulted in significantly decreased MDTs compared with the rIndo parent virus and had a similar MDT compared to the wt Indo virus. Chickens inoculated with wt Indo, rIndo/NP-184K and rIndo/NS-148E/NP-184K also presented similar histological lesions in tissues at 1 dpi, including diffuse interstitial pneumonia in the lung, moderate tracheitis, multifocal splenic necrosis, moderate cardiac degeneration, and multifocal nonsuppurative encephalitis, among others (Table 3). AIV antigen was

**Table 3** Severity of histological lesions and distribution of viral antigen in tissues from chickens inoculated with recombinant H5N1 HPAI viruses

| Tissue | A/Ck/Indo/7/03 | rIndo | rIndo/NP22A | rIndo/ NP-184K | rIndo/ NP400R | rIndo/ NP406V | rIndo/ NP423S | rIndo/ NS-148E | rIndo/NS-148E/ NP-184K |
|---|---|---|---|---|---|---|---|---|---|
| Trachea | ++/++ | ++/+ | ++/+ | ++/++ | ++/+ | ++/+ | ++/+ | ++/+ | ++/++ |
| Lung | +++/++ | ++/+ | ++/+ | +++/++ | ++/+ | ++/+ | ++/+ | ++/+ | +++/++ |
| Heart | ++/++ | −/+ | −/+ | ++/++ | −/+ | −/+ | −/+ | −/+ | ++/++ |
| Brain | ++/++ | −/− | −/− | ++/++ | −/− | −/− | −/− | −/− | ++/++ |
| Pancreas | ++/++ | −/+ | −/− | ++/++ | −/+ | −/+ | −/+ | −/+ | ++/++ |
| Adrenal | ++/++ | −/+ | −/+ | ++/++ | −/+ | −/+ | −/+ | −/+ | ++/++ |
| Intestine | +/+ | −/+ | −/+ | +/+ | −/− | −/+ | −/− | −/+ | +/+ |
| Liver | +/++ | −/+ | −/+ | +/+ | −/+ | −/+ | −/+ | −/+ | +/+ |
| Kidney | +/+ | −/+ | −/+ | +/+ | −/− | −/+ | −/+ | −/+ | +/+ |
| Spleen | +++/+++ | −/+ | −/+ | +++/+++ | −/+ | −/+ | −/+ | −/+ | +++/+++ |
| Bursa | ++/+++ | −/− | −/− | ++/++ | −/− | −/− | −/− | −/− | ++/++ |
| Thymus | +/++ | −/+ | −/+ | +/+ | −/+ | −/+ | −/+ | −/+ | +/+ |
| Muscle | +/+ | −/− | −/− | −/+ | −/− | −/− | −/− | −/− | −/+ |
| Gizzard | +/++ | −/− | −/− | −/++ | −/− | −/− | −/− | −/− | −/++ |

Tissues were taken 1 dpi and were immunohistochemically stained with antibodies to avian influenza virus nucleoprotein to visualize the viral antigen (histological lesions/viral antigen staining)

Lesions were scored as follows: − = no lesions; + = mild; ++ = moderate; +++ = severe lesions. The intensity of viral antigen staining in each section was scored as follows: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread staining

present in blood vessel endothelial cells in most tissues, tissue macrophages, cardiac and skeletal muscle myocytes, neurons and glial cells in the brain, pancreatic accinar cells, respiratory epithelium of tracheas, and adrenal corticotrophic cells. In contrast, chickens inoculated with any of the other recombinants, including rIndo and rIndo/NS-148E, presented lesions only in the trachea and lungs, and viral antigen was primarily detected in cells of these tissues and infrequently in other tissues (Table 3). Therefore, NP-184K alone or in combination with NS-148E restored the MDT of the wt Indo virus and induced similar pathology.

None of the remaining NP mutations (22, 400, 406 and 423) led to MDT or pathological changes that were significantly different from those of the rIndo group and therefore were not further studied (Tables 2, 3). Virus names are simply referred to by the amino acids changed in the remaining figures.

## A lysine residue at amino acid 184 of the nucleoprotein restores virus replication of the rIndo virus to wt Indo levels in tissues

After establishing that the NP-184K amino acid change can result in alterations of MDT and survival of infected chickens, replication of these viruses in the lung and spleen tissue was studied at 1 dpi (Fig. 1). The titers of the viruses in the tissues were determined by titration of tissue homogenates on DF-1 cells and scoring for CPE. In the lungs, wt Indo virus infection resulted in a virus titer of 6.86 $\log_{10}$ $TCID_{50}$/g tissue, while rIndo infection resulted in a significantly lower virus titer at 2.5 $\log_{10}$ $TCID_{50}$/g tissue. The NS-148E mutation did not significantly affect the virus titer of the rIndo parent virus. However, the NP-184K mutation alone (5.67 $\log_{10}$ $TCID_{50}$/g tissue) or in combination with NS-148E (6.42 $\log_{10}$ $TCID_{50}$/g tissue) resulted in significantly higher virus titers, comparable to the titer seen with wt Indo infection. In the spleen, virus titers were consistent with those found in the lungs. The wt Indo, rIndo/NP-184K, and rIndo/NS-148E/NP-184K viruses resulted in elevated virus titers, and the rIndo and rIndo/NS-148E viruses resulted in significantly lower titers than the wt Indo group.

Replication of the rIndo/NP-184K and rIndo/NS-148E/NP-184K viruses in CEFs (Fig. 2) was similar to the wt Indo virus replication, while the rIndo and rIndo/NS-148E viruses had decreased replication when compared with the wt Indo replication levels. All viruses reached similar final titers in the CEFs by the 48 h time point (Fig. 2). Therefore, the amino acid at position 184 of the NP protein affects virus replication in both the spleen and lung as well as in CEFs.

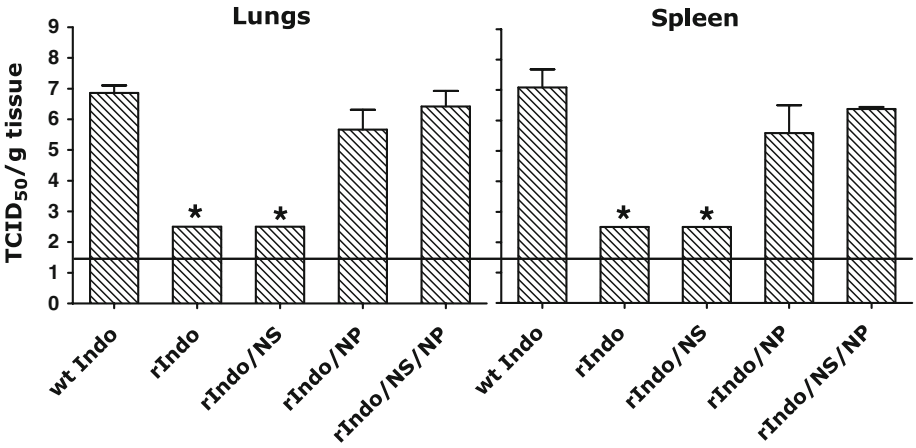


**Fig. 1** Virus titers in the lung and spleen of chickens infected with recombinant viruses. DF-1 cells were incubated with dilutions of 10% homogenates (w/vol) of spleen and lung samples from three birds per group, in triplicate. The presence of cytopathic effects was used to determine if the samples were positive for virus. $TCID_{50}$ values were determined by the method of Reed and Muench [38]. The detection limit for the assay is 1.5 $\log_{10}$ $TCID_{50}$/g tissue. Values are mean ± SEM ($n$=3). Groups marked with a *asterisk* are statistically different from the wt Indo group. Wt Indo = wild-type A/Ck/Indonesia/7/03, rIndo = recombinant A/Ck/Indonesia/7/03, rIndo/NS = rIndo/NS-148E, rIndo/NP = rIndo/NP-184K, rIndo/NS/NP = rIndo/NS-148E/NP-184K

## Amino acid 184K of NP and 148E of NS affect regulation of gene expression by the host

Previously, we showed that differences in pathogenicity of HPAI viruses can result in differential expression of several immune-related genes of the host in response to infection with the viruses [51]. Therefore, we chose to study further the effect of infection with the recombinant HPAI viruses on gene expression of *Mx1* and the cytokines IFN-α and IFN-γ, all of which were previously identified as being potentially important factors in viral infections. Figures 3 and 4 show the gene expression levels of host genes in the lungs and spleen, respectively. *Mx1* and IFN-γ gene expression levels were both up-regulated over the levels of the controls upon infection with rIndo/NP-184K or rIndo/NS-148E/NP-184K. The gene expression levels induced were similar to levels achieved with wt Indo infection and were consistent in the lung and spleen tissue. Gene expression levels of IFN-α were up-regulated after infection with the wt Indo, rIndo/NP-184K, and rIndo/NS-148E/NP-184; however, rIndo/NS-148E infection also resulted in up-regulation of IFN-α in both the lung and spleen tissue, but to a lesser extent than the up-regulation resulting from infection with the other viruses. These results suggest that differential expression of the host's genes may play a role in the differences in pathogenicity that we observed.

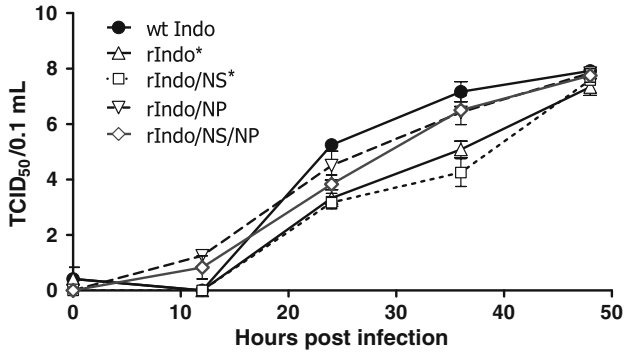


**Fig. 2** Virus replication kinetics in chicken embryo fibroblasts. Cells were infected with virus at an MOI of 0.005. Cell supernatants were harvested at 0, 12, 24, 36, and 48 h after infection, and virus titers in the supernatants were determined by CPE. Values are mean ± SEM ($n$=3). Groups marked with a *asterisk* are statistically different from the wt Indo group for that time point. Only the 24- and 36-h time points were analyzed. Wt Indo = wild-type A/Ck/Indonesia/7/03, rIndo = recombinant A/Ck/Indonesia/7/03, rIndo/NS = rIndo/NS-148E, rIndo/NP = rIndo/NP-184K, rIndo NS/NP = rIndo/NS-148E/NP-184K

## Increased levels of nitric oxide synthase result in increased nitric oxide levels with the NP-184K mutation

The levels of iNOS expression were up-regulated in lung and spleen tissues over the controls after rIndo/NP-184K or rIndo/NP-184K/NS-148E infection, to a similar extent than after wt Indo infection (Fig. 5a), again confirming that NP-184K is key to the pathogenicity of wt Indo virus.

In order to confirm the increased iNOS gene expression determined by semi-quantitative RT-PCR, we also measured the amount of nitric oxide production in the sera of chickens resulting from infection with our recombinant viruses (Fig. 5b). At 1 dpi, serum was collected from the chickens, and the total amount of nitrate + nitrite, the final end products of nitric oxide, were measured as an indication of NOS activity. The results in Fig. 5b show that control chickens had very low levels of NO in their serum,

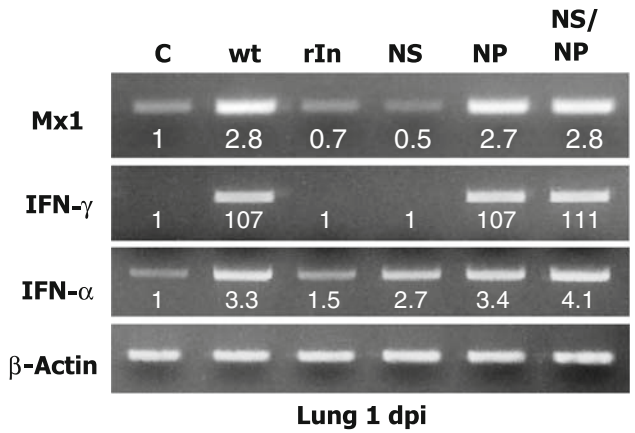


**Fig. 3** Semi-quantitative mRNA gene expression analysis in lung tissue from infected chickens 1 dpi. Total cellular RNA from lung tissue of four chickens was extracted, and equal amounts of total RNA from the chickens were pooled prior to RT-PCR analysis ($n = 1$). Analyses were carried out using different primer sets with $\beta$-actin as an amplification and loading control. Band intensities were normalized to the $\beta$-actin control for each sample, and control values were set to 1. C = control, wt = wild-type A/Ck/Indonesia/7/03, rIn = recombinant A/Ck/Indonesia/7/03, NS = rIndo/NS-148E, NP = rIndo/NP-184K, NS/NP = rIndo/NS-148E/NP-184K

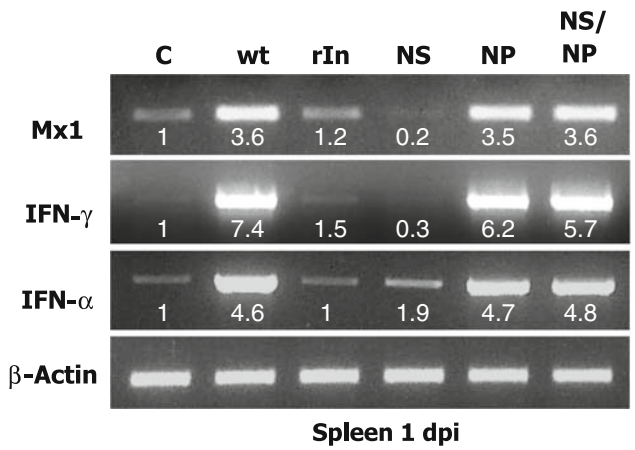


**Fig. 4** Semi-quantitative mRNA gene expression analysis in spleen tissue from infected chickens 1 dpi. Total cellular RNA from spleen tissue of four chickens was extracted, and equal amounts of total RNA from the chickens were pooled prior to RT-PCR analysis ($n = 1$). Analyses were carried out using different primer sets with $\beta$-actin as an amplification and loading control. Band intensities were normalized to the $\beta$-actin control for each sample, and control values were set to 1. C = control, wt = wild-type A/Ck/Indonesia/7/03, rIn = recombinant A/Ck/Indonesia/7/03, NS = rIndo/NS-148E, NP = rIndo/NP-184K, NS/NP = rIndo/NS-148E/NP-184K

while the wt Indo virus had higher overall NO levels. Infection with the rIndo or rIndo/NS-148E also resulted in NO levels similar to those in the control birds, indicating that at 1 dpi, these viruses did not induce NOS to significant levels. These NO measurements correlated well with the gene expression levels of iNOS in the lung tissue, as shown in Fig. 5a, and infection with rIndo and rIndo/NS-148E resulted in little gene expression change from the

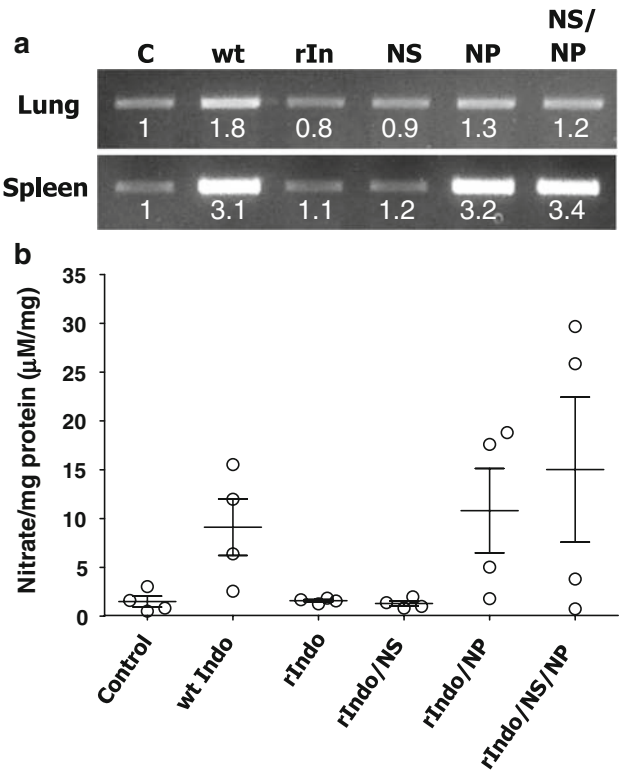


**Fig. 5** Inducible nitric oxide synthase gene expression in lung and NOS assay in serum samples from infected chickens 1 dpi. **a** Total cellular RNA from lung and spleen tissue of four chickens was extracted, and equal amounts of total RNA from the chickens was pooled prior to RT-PCR analysis ($n = 1$). Analyses were carried out using different primer sets with $\beta$-actin as an amplification and loading control. **b** Serum from four chickens per group was filtered through a 10-kDa ultrafilter and assayed for the NO end products; nitrate + nitrite and total protein were also measured. Values are mean ± SEM ($n = 4$), and values were standardized to the amount of total protein in the filtered serum. C = control, wt = wild-type A/Ck/Indonesia/7/03, rIn = recombinant A/Ck/Indonesia/7/03, NS = rIndo/NS-148E, NP = rIndo/NP-184K, NS/NP = rIndo/NS-148E/NP-184K

control levels. The rIndo/NP-184K and rIndo/NS-148E/NP-184K viruses showed an overall trend toward increased NO levels, similar to those induced by wt Indo infection, albeit with bird-to-bird variation of measurements. Again, consistent with the NO measurements in the sera, rIndo NP-184K and rIndo/NS-148E/NP-184K had up-regulated iNOS gene expression levels in the lungs and spleens of infected chickens.

## Discussion

We have identified a single amino acid change of residue 184 of the H5N1 HPAI virus nucleoprotein that restores virus replication, host gene expression and pathogenicity to wt virus levels to a variant of the virus created by reverse genetics that had impaired replication and decreased

pathogenicity in chickens. A single change of an alanine to a lysine residue at amino acid 184 of NP resulted in increased replication and pathogenicity of the viruses in chickens and also resulted in increased iNOS expression and NO levels and increased expression of the IFN-α, *Mx1*, and IFN-γ host genes, which may be involved in increased pathogenicity.

Previous research has identified many domains of the NP protein that contribute to self-association, interaction with PB2 [4, 33] and PB1 [4, 37], the presence of NLS [49] and nuclear accumulation signals [35], and RNA-binding capabilities [12, 31]. Crystallization studies with influenza A virus NP revealed potential RNA-binding pockets consisting of several positively-charged amino acids, including NP-184K [53]. The positive charge of the NP of wt Indo and NP-184K resulting from the presence of a lysine may have allowed for more efficient binding of the negatively-charged RNA, allowing more efficient replication, explaining the decreased replication seen with rIndo infection at 1 dpi, in which the positively-charged lysine residue was replaced by an alanine residue with no positive charge. The increased replication efficiency in a short time may have overwhelmed the chicken's immune system, whereas rIndo virus infection resulted in less replication, possibly allowing the immune system more time to combat the infection. This would explain the observation that the chickens infected with rIndo and rIndo/NS-148E had lower mortality and that rIndo/NS-148E infection resulted in all birds becoming sick but several eventually recovering from infection. Furthermore, numerous other studies have found NP to have many binding partners and cellular interactions that may also play a role in altering the pathogenicity of the viruses. One of these, importin-α, interacts with an identified NLS. While NP 184 is not assigned to either of the two NLS regions, another point mutation, N319K, also not in the NLS regions, has been shown to affect binding of NP to importin-α1 in mammalian cells [15]. Therefore, NP 184, if also involved in importin-α binding, could result in decreased transfer to the nucleus, resulting in slower replication, decreasing the pathogenicity of the virus. It is important to note that although the NP-184 mutation increased the replication and pathogenicity of the rIndo virus, the rIndo virus still caused mortality, and therefore the function was not completely removed by the mutation in NP-184. The precise function of the NP-184 amino acid change has on the interactions of the virus with other cellular components remains to be elucidated.

Several different host genes can be differentially regulated in response to viral infections, and many viruses have evolved mechanisms to avert these host defenses. Increased IFN-α has been shown to have antiviral effects against influenza viruses [19, 30], and the suppression of IFN-α induction is one mechanism believed to assist some AIVs in replicating in their host. Here, we have shown that the altered gene expression of the host genes IFN-α, IFN-γ, *Mx1* and iNOS in response to infection with the wt Indo and NP-184K-containing viruses was still not enough to counteract the rapid replication rates of these viruses. Evidence that NS1 is involved in the regulation of the interferon response is apparent in several mammalian species and also in chickens, in which mutations [27, 43] and deletions [7, 30, 45] have been identified in the NS1 gene that result in changes in pathogenicity of influenza viruses. The amino acid change in NS1 of rIndo from the parent virus at position 148 was a promising candidate for the cause of the differences in pathogenicity observed between the wt Indo and rIndo viruses. However, this mutation has not previously been identified as resulting in significant alteration of NS1 function. Despite the potential for this candidate mutation, the only difference that we observed with rIndo/NS-148E infection was a slightly increased gene expression level of INF-α in the spleen and lung compared with rIndo virus infection at 1 dpi. However, reverting amino acid 148 back to the wt Indo sequence had no significant effects on replication or on the MDTs of the chickens after infection, indicating that the level of INF-α expression by the host is only one factor affecting pathogenicity. The elevated IFN-α levels did not seem to correlate with the morbidity/mortality outcomes of rIndo or rIndo/NS-148E infection.

The potential role of NO in viral immunity is gaining increased attention. While some studies have shown that increased NO can have antiviral effects [1, 9, 28], the large production of NO in a short period of time as the host attempts to fight off the infection can be counterproductive, resulting in damage to the host. IFN-γ has previously been shown to induce the gene expression of iNOS, resulting in production of high NO levels [41]. The rapid replication we saw with the wt Indo and rIndo/NP and rIndo/NS/NP virus infection fits with the idea that these viruses may overwhelm the host's immune system. We measured the expression of IFN-γ and iNOS and the amount of NO present in the chicken serum to help us determine if this mechanism could play a role in the pathogenicity of these viruses. Indeed, our data suggest the elevated IFN-γ, and iNOS gene expression, in addition to the rapid viral replication resulting from the NP-184K mutation, could possibly cause the chickens to produce high levels of NO, consistent with the observed overall elevated levels of NO in those infected groups. We also saw a large amount of variation in the NO levels measured among the chickens. This could result from the single time point (1 dpi) at which we measured the NO levels. All birds were dead by the next day in the wt Indo, rIndo/NP, and rIndo/NS/NP groups. Therefore, the levels may rapidly decrease before death or may have been at their peak, and it is possible that

if we had waited and taken more time points closer to the MDT of the rIndo group, they also may have shown a steep increase in NO production as well. Furthermore, the dramatic overall increase of iNOS expression and NO production by the host that resulted from infection with the most pathogenic viruses, wt Indo, rIndo/NP-184k and rIndo/NS-148E/NP-184k, suggests that it plays an important role in the pathogenicity of these viruses.

Previous studies have shown that Mx is produced in response to viral infection and has some antiviral properties in mammals [17, 22]. Of particular interest for this study are the findings that suggest Mx targets NP to prevent infection [11, 48]. However, the role of Mx1 in avian species is controversial. Some research has shown that certain chicken breeds have an S631N mutation, which has been found to be important for antiviral activity [29]. Still others have found that Mx of chickens [3] and ducks [2] do not have antiviral capabilities as the mammalian Mx proteins do. Our results showing the up-regulation of *Mx1* gene expression by the host upon infection with wt Indo, rIndo/NP-184K and rIndo/NS-148E/NP-184K demonstrates that the level of *Mx1* gene expression in chickens changes in response to infection and suggests that even if our chicken breed does not posses a Mx1 with antiviral properties, it may play a role in the process of influenza virus infection, or it may be produced in response to increased IFN-$\alpha$ expression. It is evident that more information on the function of Mx1 in avian systems is needed for the role of NP and Mx1 in infection to be resolved.

Due to the importance of NP in influenza virus replication, NP provides a potentially valuable target for the control of influenza virus infections. Several studies have already shown that targeting NP using siRNA [20, 21, 47, 54] or morpholinos [16] is an effective method to reduce the replication of influenza viruses. Our studies suggest that amino acid 184 of the NP also is a valuable candidate for targeting siRNAs, peptide interference or antivirals.

When the amino acid sequences of the rIndo and wt Indo viruses were compared, a mutation of an isoleucine to valine at amino acid 194 of the HA was also seen in addition to the amino acid differences seen in the NP and NS1 proteins. This amino acid was not mutated due to our findings that the NP-184K mutation restored the pathogenicity of the rIndo virus to the level of the wt Indo virus. In addition, the conversion of an isoleucine to valine at amino acid 194 of HA still results in a hydrophobic amino acid at this position. Amino acid sequence alignments with other H5N1 AIVs showed that the NP 184A mutation in the rIndo virus we generated was not found in other influenza viruses. This study, however, shows that this and, potentially, other mutations in NP can have biologically relevant outcomes and are therefore useful in the study of AIV pathogenicity.

The results of the current study indicate that a single mutation in the NP protein is sufficient to increase the pathogenicity of viruses containing the NP-184K mutation. Despite the up-regulation of IFN-$\gamma$, IFN-$\alpha$, *Mx1* and iNOS by the host in response to infection with wt Indo, rIndo/NP-184K and rIndo/NS-148E/NP-184K, these innate immune response defenses were not sufficient to curb the infections in chickens, possibly due to the overwhelming replication of these viruses. While much emphasis has been placed on the roles of HA, NA and NS1 in avian influenza virus pathogenicity, these data raise the question as to whether or not spontaneous single mutations in other virus genes may significantly affect pathogenicity of avian influenza viruses.

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